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Specification, as originally filed, with Application for Patent Serial No: **2,450,000**, on December 18, 2003, by **ALBERTA RESEARCH COUNCIL INC.,** assignee of Salehuzzaman Shah and Randall Weselake, for "Method of Creating Plants with Reduced Level of Saturated Fatty Acid in Seed Oil".

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METHOD OF CREATING PLANTS WITH REDUCED LEVEL OF SATURATED FATTY ACID IN SEED OIL

ABSTRACT

The present invention provides transgenic plants with reduced level of saturated fatty acid in the seed oil and methods of making these plants. The transgenic plants developed through this method contain reduced level of saturated fatty acid in seed oil due to expression of a gene ('desaturase gene'), that increases the unsaturated fatty acid content of the plant's cell, operably linked with a signal for protein retrieval and retention in the endoplasmic reticulum. Equivalent transgenic plants transformed with only the desaturase gene, without the retrieval and retention signal, had no effect on the content of saturated fatty acids in seed oil. One example of the invention is a plant expressing a heterologous delta-9 desaturase gene from cyanobacterium *Anacystis nidulans*, which particularly converts lipid-bound 16:0 and 18:0 fatty acids into corresponding 16:1 and 18:1, respectively.

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METHOD OF CREATING PLANTS WITH REDUCED LEVEL OF SATURATED FATTY ACID IN SEED OIL

BACKGROUND OF THE INVENTION

There has been significant interest in altering fatty acid (FA) synthesis to create vegetable oils designed for specific purposes. The properties of the oil are determined by its fatty acid composition. This can affect both nutritional composition and oxidative stability. Canola (*Brassica napus*) oil is traditionally low in saturated fatty acid (7%) although there has been increasing pressure in the market to lower this level even further. The main component of saturated fatty acid in most vegetable oil is 16:0 (palmitic acid) and 18:0 (stearic acid). The level of saturated FAs in various types of fats and oils is a major health concern. Lower content of the saturated FAs is desirable. Applicant has successfully reduced the 16:0 and 18:0 fraction of fatty acids from seed oil with consequent reduced total saturated fatty acid level.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In one aspect, the present invention provides a signal for protein retention and retrieval to endoplasmic reticulum, comprising a nucleotide sequence that encodes amino acids KDEL or KKXX, wherein X is any amino acid found in protein molecules.

In another aspect, the present invention provides a DNA sequence operably linked with a signal for protein retention and retrieval to endoplasmic reticulum and capable of expressing an enzyme with the activity of a fatty acid desaturase.

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In yet another aspect, the present invention provides a plant cell containing the above mentioned DNA sequence.

In a further aspect, the present invention provides a vector comprising the above mentioned DNA sequence, capable of delivering said DNA sequence into a plant or plant cell.

In yet another aspect, the present invention provides use, as a source of food low in unsaturated fatty acids, of a plant comprising stably inserted into its genome the above mentioned DNA sequence.

As an example of the invention, applicant developed canola plants with almost 50% reduced saturated FA level compared to current commercial cultivars. This was achieved by introducing a desaturase gene operably linked to an endoplasmic reticulum (ER) retrieval and retention signal. Applicant has demonstrated that using a desaturase gene construct without the ER retrieval and retention signal had no effect in reducing saturated fatty acid level in canola seed oil. The level of saturated FA was reduced only when an ER retrieval and retention signal was operably linked to a desaturase gene. Compared to 7.4% saturated FA in canola, applicant has developed lines containing as low as 3.5% saturated FA. Both major saturated FAs (16:0 and 18:0) were reduced in these lines.

In a preferred embodiment, des9 (Ishizaki-Nishizawa et al. 1996) from a blue-green algae (cyanobacterium, *Anacystis nidulans*) was used. The des9 desaturase gene encodes a protein that introduces a *cis*-double bond (desaturates) at the delta-9 position of

saturated fatty acids bound to lipids. The protein has higher specificity for 16:0 fatty acids but also desaturates 18:0 fatty acids. The cyanobacterial des9 desaturase, which is also sometimes referred to as DSG gene, is described in detail in U.S. Pat. No. 6,043,411 to Nishizawa et al.; in Nature Biotechnology 14: 1003-1006 and registered in EMBL GeneBank as accession number X77367, all of which references are incorporated herein by reference.

Many transmembrane proteins are processed and transported to the cell surface. Some of these proteins can be retrieved and retained in the ER by adding a signal sequence to the protein. Examples of suitable signal sequence are KKXX, wherein X is any amino acid (Vincent et al. 2003) and KDEL. The signal sequence may be added by genetic engineering of the gene that codes for the protein. Oil synthesis and desaturation of the lipid bound fatty acids take place in the ER of the cells in seeds. Applicant has demonstrated in the present invention that a desaturase gene (such as des9 from cyanobacterium) functions properly to reduce saturated fatty acids only in combination with a retrieval and retention signal. However the scope of this invention is not limited to des9 nor to any particular signal sequence, and is applicable to other desaturase proteins as well as to other signal sequences which result in the retrieval and retention in the ER of desaturase proteins.

In one embodiment for reduction of saturated fatty acid in seed oil, applicant assembled a DNA construct, which contained two expression cassettes. The term DNA construct refers here to a genetic DNA sequence used to transform plant cells from which transgenic plants are being

generated. The term expression cassette refers here to a sequence of DNA comprised of a coding region to which promoter and terminator regulatory sequences have been linked at the 5′ and 3′ end to achieve proper expression of the gene as well as the gene product in the transformed plant.

In this embodiment, a first cassette comprises the des9 gene of cyanobacterium operably linked to the signal KKSS and fused to the seed specific napin promoter from *Brassica* and the rbcs3′ transcription terminator from pea. In the present embodiment, applicant used the powerful seed-specific storage protein napin promoter, however other seed-specific promoters contemplated for use in the present invention include, but are not limited to: cruciferin and hydroxylase. In the present example, the coding region is also operably linked at the 3′ end with the rbcs3′ transcription terminator as a regulatory sequence. Other useful 3′ regulatory regions which can also be used in the present invention include, but are not limited to: nopaline synthetase polyadenylation region (NOS) and octopine polyadenylation region (OCS). In the signal sequence, the symbol 'S' stands for the amino acid serine. Although applicant has used this amino acid downstream of KK, any other amino acids can be used instead of 'S' to retrieve and retain the protein in the ER. The term 'operably linked' means that the regulatory sequences necessary for expression of the coding sequences and the ER retrieval and retention signal sequences are placed in the DNA construct in the appropriate position relative to the coding sequence and in correct reading frame so as to effect expression of the gene.

In this embodiment, the second expression cassette comprises a promoter, coding region and terminator expressing a gene product suitable to aid in the identification and selection of transformed plant cells and plants. The second expression cassette is optional, as other methods may be used to identify and select transformants.

In one embodiment, the DNA construct was introduced into the genome of the canola plants using Agrobacterium T-DNA mediated plant transformation. Transgenic plants expressing the DNA construct can also be generated using other methods of DNA delivery known to those skilled in the art. These include, but are not limited to: biolistic DNA delivery, electroporation of protoplasts, direct DNA uptake, PEG treatment of protoplast, UV laser microbeam, Gemini virus vectors, liposome-mediated DNA uptake, calcium phosphate treatment of protoplasts, agitation of cell suspensions with microbeads coated with the transforming DNA, and the like, all of which are also contemplated to be within the scope of the present invention.

Using the Agrobacterium binary vector system, the transformation of plant nuclei was accomplished by: a) inserting the des9 gene and the retrieval and retention signal KKSS into the vector, b) co-cultivation of appropriate plant tissue with a suspension of recombinant Agrobacterium followed by incubation in non-selective medium, c) transfer of the plant tissues into selective medium to identify transformed tissue, and d) identification of transformants regenerated to intact plants. The amount of expression of the transgenes can vary depending on the

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position and number of their insertion into the nuclear genome. Therefore, several transformants should be regenerated and tested for expression of the transgene and for altered fatty acid profile.

Transformation with the cyanobacterial delta-9 desaturase operably linked with the ER retrieval and retention signal resulting in reduction of total saturated fatty acid content in seed oil has been demonstrated in canola (*Brassica napus*). The biochemistry of oil synthesis, desaturation of fatty acids and sub-cellular localization of these metabolic reactions in other oil seed crops are either the same or similar to that in canola. Therefore, the discoveries made in accordance with the present invention are expected to be applicable to any oil seed plants, such that all plants are contemplated for use in this invention. These include, but are not limited to, all dicotyledonous and monocotyledonous flowering plants like soybean, corn, sunflower, olive, palm, coconut, safflower, flax, etc.

The present invention is also intended to encompass cells and tissues of the aforementioned transgenic plants. In a preferred embodiment, seeds of the transgenic plants produced by the method of invention are provided. The plants grown from the aforementioned seeds or other reproductive units can be used in crosses and selection methods to transfer the gene of interest into other genotypes, cultivars, varieties and the like, through breeding and selection. Thus a great variety of plants carrying the DNA construct can be produced with reduced saturated fatty acid content and also those plants are intended to be encompassed in the present invention.

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TABLE:

Fatty acid content (mol %) of seeds of transgenic canola plants carrying cyanobacterial des9 gene linked with ER retrieval and retention signal (C8-19.1), transgenic plant carrying only the des9 gene (C7-15) and non-transformed plants (WT).

	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	Saturated
WT	4.07	0.16	1.87	69.08	15.67	6.21	0.70	1.53	7.21
C7-15	4.03	0.37	1.85	63.82	18.16	8.11	0.93	2.71	6.81
C8-19.1	2.10	2.46	1.34	69.07	17.63	5.98	0.30	1.18	3.74

REFERENCES:

Nishizawa O, Toguri T (1996) Gene for fatty acid desaturase, vector containing said gene, plant transformed with said gene, and process for creating said plant. U.S. patent number 6,043,411.

Nishizawa O, Fujii T, Azuma M, Sekiguchi K, Murata N, Ohtani T, Toguri T (1996) Low-temperature resistance of higher plants is significantly enhanced by a nonspecific cyanobacterial desaturase. Nature Biotechnology 14: 1003-1006.

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Vincent MJ, Martin AS, Compans RW (1998) Function of the KKXX motif in endoplasmic reticulum retrieval of a transmembrane protein depends on the length and structure of the cytoplasmic domain. J Biol Chem. 273:950-6.

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CLAIMS:

1. A signal for protein retention and retrieval to endoplasmic reticulum, comprising a nucleotide sequence that encodes amino acids KDEL or KKXX, wherein X is any amino acid found in protein molecules.

2. A DNA sequence operably linked with a signal for protein retention and retrieval to endoplasmic reticulum and capable of expressing an enzyme with the activity of a fatty acid desaturase.

3. The DNA sequence of claim 2 wherein the signal is as defined in claim 1.

4. The DNA sequence of claim 2, wherein the fatty acid desaturase is a delta-9 desaturase.

5. The DNA sequence of claim 4, wherein the delta-9 desaturase is a cyanobacterial delta-9 desaturase.

6. A plant cell containing the DNA sequence of any one of claims 2 to 5.

7. The plant cell of claim 6 selected from the group consisting of cells from monocotyledonous and dicotyledonous flowering plants that produce oil in their seeds.

8. A vector comprising the DNA sequence of any one of claims 2 to 5 capable of delivering said DNA sequence into a plant or plant cell.

9. Use, as a source of food low in unsaturated fatty acids, of a plant comprising stably inserted into its genome the DNA sequence of any one of claims 2 to 5.